Available online at www.sciencedirect.com

SCIENCE DIRECT®

Bioelectrochemistry 66 (2005) 129–138

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

Direct immobilisation of DNA probes for the development of affinity biosensors

Ilaria Mannelli[a], Maria Minunni[a], Sara Tombelli[a,*], Ronghui Wang[a,b], Maria Michela Spiriti[a], Marco Mascini[a]

[a]*Dipartimento di Chimica, Università degli Studi di Firenze, Polo Scientifico, Via della Lastruccia 3, Sesto Fiorentino, 50019, Italy*
[b]*Chemistry and Chemical Engineering College, Central South University, Changsha 410082, Hunan, PR China*

Received 19 January 2004; received in revised form 7 April 2004; accepted 13 April 2004
Available online 23 August 2004

Abstract

An immobilisation procedure based on the direct coupling of thiolated probes (Probe-C6-SH) to bare gold sensor surfaces has been compared with a reference immobilisation method, based on the coupling of biotinylated probes onto a streptavidin-coated dextran-modified surface.

The instrumentations used were a quartz crystal microbalance (QCM) and the optical instruments Biacore X™ and Spreeta™ based on surface plasmon resonance (SPR).

The performances of the DNA-based sensors resulting from direct coupling of thiolated DNA probes onto electrodes of quartz crystals or gold SPR-chips have been studied in terms of the main analytical parameters, i.e. selectivity, sensitivity, reproducibility, etc.

In particular, the two immobilisation approaches have been applied to the analysis of oligonucleotides, DNA amplified by polymerase chain reaction (PCR) and genomic DNA enzymatically digested.

Keywords: Biosensors; DNA probes; Immobilisation procedure

1. Introduction

In the last two decades, biosensors had a wide impact in clinical, food and environmental analysis. In particular, DNA-based biosensors, where a ssDNA probe is immobilised on the surface of the sensor, allow rapid, real-time monitoring of hybridisation with the target DNA. The detection relies on the biorecognition between a probe immobilised on the sensing surface and the target sequence in solution.

Probe immobilisation is a fundamental step in DNA-based biosensor development. In the present work, a comparison between an immobilisation procedure based on the direct coupling of thiolated probes (Probe-C_6-SH) to bare gold sensor surfaces [1,2] and a reference immobilisation method, based on the coupling of biotinylated probes to a streptavidin-coated sensor [3] is shown. This latter immobilisation method has been extensively applied in many previous works [4–16]. This method resulted very efficient in terms of sensitivity, selectivity and stability of the realised sensors for hybridisation detection [8,10,16,17]. More recently, chemisorption of DNA probes onto transducer surfaces based on the formation of gold-thiol bonds has been reported by some authors [2,18–23]. Since the direct coupling of probes by self-assembly of terminally thiol-labelled oligonucleotides is very suitable for piezoelectric and surface plasmon resonance (SPR) based sensors, in this work, both these transduction techniques have been examined.

The hybridisation detection has been studied with a quartz crystal microbalance (QCM) (Seiko QCA922) and two SPR devices (Biacore X™ and Spreeta™). Each

* Corresponding author. Tel.: +39 0554573323; fax: +39 0554573397.
E-mail address: sara.tombelli@unifi.it (S. Tombelli).

1567-5394/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2004.04.008

instrument provides a gold surface, consisting of a quartz crystal with gold electrodes evaporated on both sides, for the QCM device, and of a glass slide coated with vaporised gold, for the SPR systems. The application of the same coupling strategy to these different devices was conducted with the aim of exploiting its applicability to different instrumentations using different sensor formats.

The different systems have been applied to the analysis of DNA amplified by polymerase chain reaction (PCR) and genomic DNA enzymatically digested. In particular, the amplified fragment was a region of the 35S promoter, a marker widely used for genetically modified organisms analysis. The target sequence in the genomic DNA is internal to a highly repeated fragment of bovine genome. Highly repeated DNA fragments are specific sequences, of various length (from a few to thousands of nucleotides), which are repeated in the genome and can correspond to a high percentage of this (around 30%).

2. Experimental

2.1. Apparatus and reagents

The QCM device was based on 10 MHz At-cut quartz crystals (14 mm) with gold electrodes (42.6-mm^2 area) evaporated on both sides (NuovaMistral, Latina, Italy). The quartz resonator was housed inside a methacrylate cell such that only one side of the crystal was in contact with the solution in the cell well. The frequency variations were continuously recorded using a quartz crystal analyser (Models QCA922, Seiko EG&G, Chiba, Japan). The frequency shifts reported in the paper are the difference between two stable values ($\pm$1 Hz).

The SPR device Biacore X™ (Biacore, Uppsala, Sweden) and a dextran-modified sensor chip (CM5) or a bare gold sensor chip (SIA kit Au), the SPR device Spreeta™ (Texas Instruments, USA) and a bare gold Spreeta sensor were used for the SPR experiments. All the measurements were conducted at a flow rate of 5 μl/min and 25 °C.

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), 6-mercapto-1-hexanol (MCH) and streptavidin were purchased from Sigma Aldrich (Milan, Italy). *N*-hydroxysuccinimide (NHS) was obtained from Fluka (Milan, Italy). Other reagents for the buffers were purchased from Merck (Darmstadt, Germany).

The composition of the buffers and solutions used for the experiments is as follows:

- Immobilisation buffer (I): NaCl 300 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4.
- Immobilisation solution (II): KH_2PO_4 1 M, pH 3.8.
- Hybridisation buffer (I): NaCl 150 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4.
- Hybridisation buffer (II): NaCl 150 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, Tween 20 0.005%, pH 7.4.
- Restriction enzyme (*Eco*RI) buffer (M-Medical, Genenco, Milano, Italy): 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 0.02% Triton X-100, 0.1 mg/ml BSA.

2.2. Samples

5′Thiolated synthetic oligonucleotides were from Sigma-Genosys (Cambridge, UK); 5′biotinylated and all the other synthetic oligonucleotides were from MWG Biotech (Florence, Italy).

The base sequences of the 5′-functionalised probes, synthetic targets and non-complementary strands are described below:

P35S	
Biotinylated probe:	5′biotin-GGCCATCGTTGAAGATGCCTCTGCC 3′
Non-specific probe (1):	5′biotin-AATGATTAATTGCGGGACTCTAATC 3′
Thiolated probe:	5′HS-$(CH_2)_6$-GGCCATCGTTGAAGATGCC TCTGCC 3′
Target:	5′GGCAGAGGCATCTTCAACGATGGCC 3′
Non-complementary sequence (1):	5′GATTAGAGTCCCGCAATTAATCATT 3′
Bovine	
Biotinylated probe:	5′biotin-TCACGCAGCTCAGCAGGCCCT 3′
Thiolated probe:	5′HS-$(CH_2)_6$-TCACGCAGCTCAGCAGGCCCT 3′
Target:	5′AGGGCCTGCTGAGCTGCGTGA 3′
Non-complementary sequence (2):	5′GGCAGAGGCATCTTCAACGATGGCC 3′
Non-complementary sequence (3):	5′CAAGGGAGGGAGAGACAGAGAGGCC 3′
Blocking oligo 1:	5′GTCTGCTCATCTGCTTGACAATTTC 3′
Blocking oligo 2:	5′TAATCAAGTAGATGAGCAGGCAG 3′

The plasmidic DNA (pBI121) and the genomic DNA from genetically modified (GM) maize real samples were kindly provided by Prof. M. Buiatti, University of Florence (Italy).

Genomic bovine (*Bos taurus*) and porcine (*Sus scrofa domesticus*) DNA were commercially available from Novagen (Darmstadt, Germany).

2.3. Immobilisation of the oligonucleotide probe on gold

2.3.1. QCM device

The gold electrode surface was first cleaned with a boiling solution consisting of H_2O_2 (30%), NH_3 (30%), milliQ H_2O in a 1:1:5 ratio. The resonators were dipped in the solution for 10 min. They were then thoroughly washed with distilled water.

Two different procedures were employed for probe immobilisation. The first was a multi-steps procedure based on the interaction between a biotinylated probe and the

streptavidin on the surface. This procedure was used here as reference method in the comparison with the second one that is based on the direct chemisorption of thiol-derivatised probes.

2.3.1.1. Biotin-derivatised probe procedure. The gold sensor surface was modified by formation of a carboxylated dextran layer, for the immobilisation of streptavidin [3], on a 11-mercaptoundecanol self-assembled monolayer. Then the biotinylated oligonucleotide (1 μM, P35S probe, and 2 μM, bovine probe, in immobilisation buffer) was immobilised [24–26].

2.3.1.2. Thiol-derivatised probe and blocking thiol procedure. The gold sensor surface was modified with a thiol-derivatised probe and a blocking thiol [2,26–30].

The resonator, after the cleaning step, was thoroughly washed with distilled water and left in ethanol overnight. Then the resonator is inserted in the cell and treated with a solution (1 μM) of thiolated probe in immobilisation solution for 2 h. After washing with immobilisation solution and milliQ H_2O, 200 μl of blocking thiol solution (MCH, 1 mM) was injected into the cell and the reaction was allowed to proceed for 1 h, before the final washing with milliQ H_2O.

2.3.2. SPR device

2.3.2.1. Biotin-derivatised probe procedure. This immobilisation procedure was conducted on the Biacore X™ device as reported in Refs. [15] and [16].

The dextran-modified sensor chip (CM5) was directly docked into the Biacore X™ instrument. Thirty five microliters of a solution containing 50 mM NHS and 200 mM EDAC in water was injected to activate the dextran-modified surface. The chip was further modified with streptavidin (200 μg/ml in acetate buffer 10 mM, pH 5.0). Then, the residual reacting sites were blocked with 35 μl solution of ethanolamine hydrochloride (pH 8.6, 1 M water solution). Finally, the biotinylated probe was added (100 μl probe 1 μM in immobilisation buffer (I)).

The presence of two flow-cells on the sensor chip allows the immobilisation of two different probes. The probe in cell 1 provides the complementary counterpart for the target of interest, while the non-specific probe in cell 2 was only used as a control surface. Both probe sequences were immobilised using the same protocol.

2.3.2.2. Thiol-derivatised probe and blocking thiol procedure. Biacore X™: the gold sensor chip was cleaned with a solution consisting of H_2O_2 (30%), NH_3 (30%) and milliQ water in a 1:1:5 ratio for 10 min and then thoroughly washed with milliQ water. After the cleaning step, the sensor chip was covered with a solution (1 μM, 1 ml) of thiolated probe in immobilisation solution (II) and incubated at room temperature for 2 h. Following incubation, the sensor chip was washed with milliQ water and then it was treated with 1 mM (1 ml) blocking thiol solution (MCH) at room temperature for a further hour in the dark. After washing with water, the chip was docked into the Biacore X™ instrument, ready for hybridisation.

Spreeta™: the gold Spreeta™ sensor surface was firstly cleaned with ethanol using a lens paper to remove fingerprints, oily residues and dust particles, and then a further cleaning step has been done using the same cleaning solution used for the Biacore X™ chip. After the cleaning step, the Spreeta™ sensor was put into the instrument IFC (Integrated Flow Cell). The coating steps comprised 2-h running with the thiolated probe (1 μM) and then 1-h running with the blocking thiol solution (MCH, 1 mM) at a flow rate of 5 μl/min and at 25 °C.

2.4. Hybridisation with synthetic oligonucleotides

2.4.1. QCM device

The oligonucleotide target complementary to the immobilised probe was used for the characterisation of the biosensor.

The hybridisation with the target oligonucleotide was performed adding 100 μl of the oligonucleotide solution in hybridisation buffer to the cell well. The reaction was monitored for 10 min and then the surface washed with the hybridisation buffer to remove the unbound oligonucleotides. We report the frequency shift as the difference between this final value and the value displayed before the hybridisation reaction. Both frequency values are taken when the crystal is in contact with the same buffer solution (hybridisation buffer) so the shift is due only to compounds fixed on the gold surface during the reaction [24–26,31].

In all the experiments, the single stranded probe was regenerated by two consecutive treatments of 30 s with 1 mM HCl allowing a further use of the sensor.

All the experiments were performed at room temperature.

2.4.2. SPR device

Hybridisation experiments were conducted in the Biacore X™ at a flow rate of 5 μl/min and at 25 °C injecting 25 μl of the sample solution on the modified chip. The reaction was monitored for 5 min and then the sensor chip was automatically washed with hybridisation buffer to remove the unbound DNA material. The analytical signal, reported as Resonance Units (RU), was derived from the difference between the final value and the value recorded before the target injection (baseline). The single-stranded probe was regenerated by 1 mM HCl (streptavidin–biotin method) or 2.5 mM HCl (thiol-derivatised probe method).

Using the Spreeta™ instrument, hybridisation measurements consisted of 5-min hybridisation, 30 s of running with hybridisation buffer to remove the unbound DNA material and finally 2 min of regeneration with 2.5 mM HCl to go back to the baseline (flow rate 5 μl/min, T=25

°C). The analytical signal, reported as Refractive Index (RI), was derived from the difference between the value recorded before (baseline) and after the hybridisation when the sensor surface is in contact with the same buffer solution.

2.5. Sample extraction and amplification

The plasmidic (pBI121) DNA and the genetically modified (GM) maize DNA were extracted using the QIAprep kit (Qiagen, Milan, Italy) and following the European screening method for GMO detection [32], respectively. The concentration of the extracted DNA material was determined spectrophotometrically at 260 nm (GeneQuant, Amersham Biosciences, Milan, Italy).

A 243-bp DNA fragment containing the target sequence P35S (25mer) was amplified by using the sense (5′ GCTCCTACAAATGCCATCATT 3′) and antisense (5′ CTCCAAATGAAATGAAC 3′) primers (MWG Biotech). The PCR conditions were as follows: initial 94 °C for 4 min, 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and 72 °C for 3 min; 35 PCR cycles. All PCR experiments were conducted with a MiniCycler, MJ Research (model PTC150) (Waltham, MA, USA).

Screening of the PCR products was performed by gel electrophoresis and visualised through a UV transilluminator.

The control solution (blank) contained all the PCR reagents except the DNA template.

2.6. Genomic DNA digestion

The genomic DNA was digested using the restriction enzyme *Eco*RI, to obtain DNA fragments of around 400–500 bp [33], containing the target sequence. By the WebCutter 2.0 software (web available), it was verified that the consensus sequence recognised by the enzyme is not present inside the *B. taurus* satellite no. 13 (highly repeated sequence, 247 bp). This is important to ensure that the fragmentation does not affect the ability of the target sequence to hybridise to the immobilised probe.

Ten micrograms of genomic DNA has been digested with two enzymatic units of *Eco*RI in restriction enzyme buffer. The reaction has been allowed to proceed at 37 °C overnight and then at 75 °C for 10 min to inactivate the enzyme. Afterwards, the digested DNA has been precipitated with 500 μl of absolute ethanol and 20 μl of sodium acetate 3 M (pH 5.2). The precipitating solution was centrifuged at 13,000 rpm for 45 min at room temperature and the supernatant removed. The DNA pellet was washed with 500 μl of ethanol 70% at −20 °C and centrifuged at 1300 rpm for 30 min; the supernatant has been removed, the pellet has been dried in vacuum for 15 min and suspended in 100 μl of sterile bi-distilled H_2O.

The reaction success has been confirmed by the electrophoresis analysis.

2.7. Denaturation of the PCR amplified DNA and the digested genomic DNA

The amplicons obtained from PCR and the DNA fragments obtained by enzymatic digestion have a double helix structure and the two strands should be separated (denatured) to allow the hybridisation with the probe immobilised on the sensor surface.

A denaturation procedure that combines the thermal dissociating effect to the steric hindrance caused by the binding of two oligonucleotide sequences to the separated DNA strands was applied. The principle of this method relies on the use of two small (20–25 bases) oligonucleotides added to the sample. One oligonucleotide is complementary to the strand containing the probe and the other one to the strand containing the target, but binds laterally so to not overlap with them. By the interaction between the thermally separated DNA strands and these oligonucleotides, re-association between dsDNA strands is prevented, and surface hybridisation can occur. After the addition of the two oligonucleotides (1 μM), the sample was incubated at 95 °C for 5 min and then 1 min at 50 °C. This second temperature is the appropriate temperature for the annealing of the oligonucleotides to the complementary DNA sequences [34].

For P35S PCR samples, the primers for the PCR procedure have been also used as blocking oligonucleotides in the denaturation; for bovine genomic DNA denaturation, blocking oligonucleotides 1 and 2 were used.

2.8. Hybridisation with the PCR amplified DNA and the digested genomic DNA

2.8.1. QCM device

The procedure for the hybridisation of PCR amplified DNA and digested genomic DNA is the same as the one described for oligonucleotides, with few exceptions due to the greater complexity of the sample. Due to this characteristic, the hybridisation reaction was allowed to proceeded for 20 min.

Moreover, in the case of digested genomic DNA, a different regeneration treatment was employed, which consisted of a first addition of alkaline solution (15 s with NaOH 100 mM), which dissociates the two strands, followed by 30 s with a solution consisting in 200 mM Tris–HCl, pH 7.0 and 0.1×SSC, 0.1%(w/v) SDS (regeneration solution).

The surface performances were controlled, using standard solutions of synthetic oligonucleotides, before and after the hybridisation–regeneration cycles.

2.8.2. SPR device

The procedure for the hybridisation of PCR amplified DNA and digested genomic DNA is the same as the one described for oligonucleotides, with the exception of the regeneration method.

Different concentrations (5, 10 and 50 mM) of HCl and NaOH were tested to find the optimal condition for the

regeneration of the surface after the interaction with the PCR amplified DNA sample.

For the regeneration of the surface after the interaction with the digested genomic DNA, a different treatment was used, which consisted of a first addition of alkaline solution (15 s with NaOH 100 mM), followed by 30 s with the regeneration solution mentioned in the previous paragraph.

3. Results and discussion

3.1. Measurements with synthetic oligonucleotides

The analytical performances of the developed sensors were tested; the sensitivity, specificity, reproducibility and stability were evaluated using standard solutions of synthetic oligonucleotides. Two different probes, P35S and bovine, were immobilised and analysed separately on the different devices.

3.1.1. QCM device

3.1.1.1. Biotin-derivatised probe procedure

Probe P35S. The calibration curve obtained with complementary oligonucleotides for the P35S probe in a concentration range of 0–0.75 μM is shown in Fig. 1 (curve 1). The curve shows a profile with a linear region in the range 0–0.5 μM and an average coefficient of variation% (CV%) of 9%. The average CV% is the mean value of the coefficients of variation% calculated for each concentration, tested in triplicate. The specificity of the interaction was tested using a solution 1 μM of non-complementary sequence 1. The signal resulting from this interaction was negligible (<3 Hz) confirming the specificity of the system. The surface was regenerated by treatment with HCl 1 mM and used for a maximum of 25 cycles (data not shown).

Probe bovine. The hybridisation between the probe and the complementary oligonucleotide was tested in a concentration range varying from 0 to 0.75 μM.

The results are shown in Fig. 2 (curve 1). A CV% of 10% has been calculated for all the concentrations. The selectivity of the crystal surface was confirmed by the absence of frequency shift with the non-complementary sequence 1, 1 μM. The surface was regenerated by HCl 1-mM treatment.

3.1.1.2. Thiol-derivatised probe and blocking thiol procedure

Probe P35S. Fig. 1 shows the calibration curves obtained with the P35S complementary oligonucleotides after immobilising the thiolated probe by the direct chemisorption on the gold electrode surface. Different probe concentrations (1 μM (curve 2) and 0.5 μM (curves 3 and 4)) and immobilisation times (2 h (curves 2 and 4) and 1+1 h (curve 3)) were tested to find the optimal immobilisation conditions. In particular, curve 3 was obtained using fresh solution of thiolated probe during the second hour. Curves 2 and 3 show a similar profile indicating a similar hybridisation efficiency of the probe 1 μM immobilised in 1 (2 h) or 2 (1+1 h) steps. On the contrary, a 0.5-μM concentration of probe resulted in a lower sensitivity of the sensor. These results are in agreement with the data reported in the literature [2].

The best conditions of immobilisation, probe concentration of 1 μM and a contact time of 2 h, were then adopted for the analysis of PCR and genomic DNA samples.

The specificity of the sensor was verified with a non-complementary oligonucleotide 1 μM. The regeneration with HCl 1 mM allowed a maximum of 20 cycles.

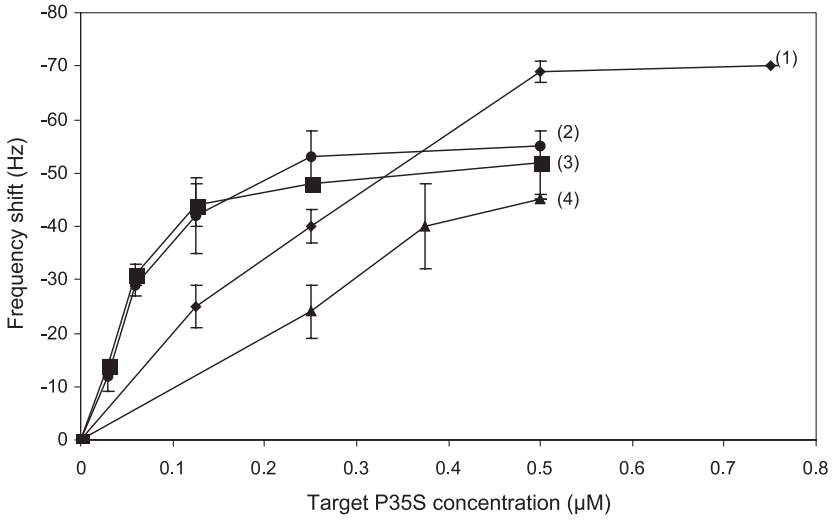

Fig. 1. Calibration curve for P35S obtained from biotin-derivatised probe immobilisation procedure (curve 1, reference procedure) and thiol-derivatised probe/blocking thiol procedure (curves 2, 3, 4) using QCM device. In particular, the curve 1 is relative to a biotin-derivatised probe 1 μM; while the curves 2, 3 and 4 were obtained using a probe concentration and a contact time of: 1 μM, 2 h (curve 2); 0.5 μM, 1+1 h (curve 3) and 0.5 μM, 2 h (curve 4).

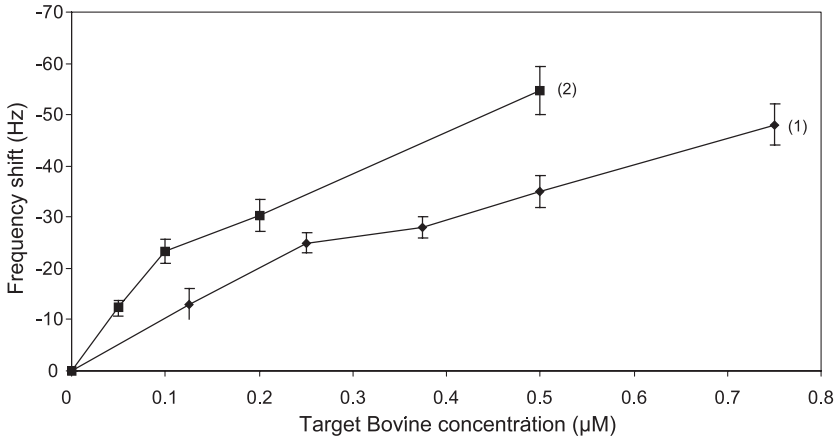

Fig. 2. Calibration curve for P35S obtained from biotin-derivatised probe immobilisation procedure (curve 1, reference procedure) and thiol-derivatised probe/blocking thiol procedure (curve 2) using QCM device. The probe concentration was 1 μM, in both cases, and the contact time, in the thiol-derivatised probe, was 2 h.

From the results reported, it is possible to observe that the dynamic range, obtained with the QCM device, is larger with biotinylated probes (curve 1, Fig. 1). Whereas, the sensitivity is higher with thiolated probes (curves 2 and 3, Fig. 1). An explanation for this phenomenon could be found performing detailed surface studies, which were not conducted in this work. Anyway the different behaviour of the two immobilised probes could be due to their different density and orientation on the surface.

Probe bovine. In this case, the range of measured concentration was 0–0.5 μM (Fig. 2, curve 2) and the non-complementary oligonucleotide used is sequence 2.

The sensor performances are similar to the previous case. The average CV% calculated is 10%. The sensor surface shows selectivity towards the target, because no frequency shift was observed with the non-complementary sequence 2, 1 μM. The surface regeneration was performed with HCl 1 mM.

3.1.2. SPR device

3.1.2.1. Biotin-derivatised probe procedure on Biacore X™

Probe P35S. Probe P35S and TNos were immobilised in cells 1 and 2, respectively. After the probe immobilisation, a calibration curve was performed using the P35S target in a 0–0.5 μM concentration range (Fig. 3). A linear relationship can be observed in the concentration range 0–0.1 μM. For each concentration, no detectable shift (ΔRU<1 RU) was obtained from cell 2 where the non-specific probe was

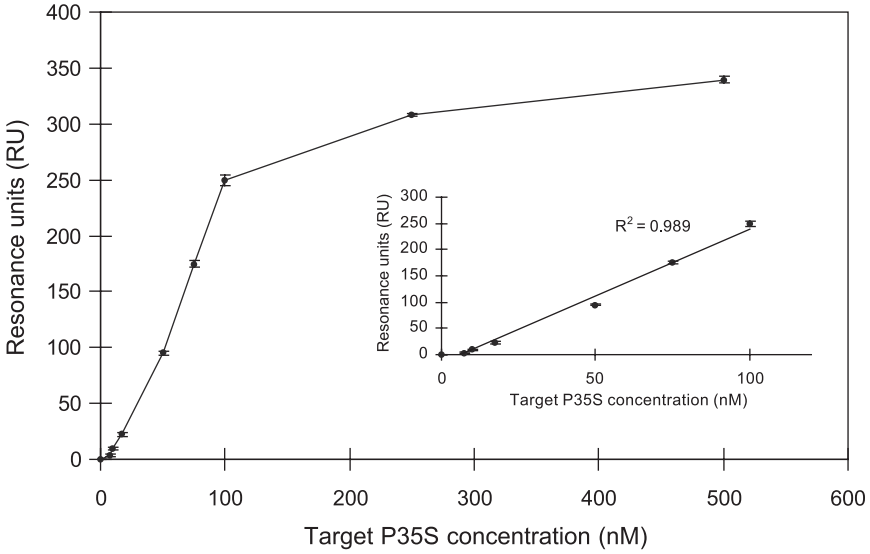

Fig. 3. Calibration curve for P35S obtained from streptavidin–biotin binding immobilisation procedure (reference procedure) using BIACORE X™ instrument and zoom of the linear region (cell 1: probe P35S; cell 2: probe TNos).

immobilised. This result confirmed the high specificity of the system when using synthetic oligonucleotides.

The reproducibility has also been estimated and using the same concentration (0.1 μM) for nine different cycles of hybridisation/regeneration on the same chip in one day, the average value was 249±12 RU, resulting in a CV% of 5%.

In all the experiments, the single-stranded probe was regenerated by a 1-min (5 μl) treatment with 1 mM HCl. The regeneration resulted to be of 92–100% with all tested target concentrations (0–0.5 μM), demonstrating the efficiency of the treatment. Such step could be performed more than 100 times without affecting the hybridisation efficiency (data not shown).

3.1.2.2. Thiol-derivatised probe and blocking thiol procedure on Biacore X™

Probe P35S. When using the immobilisation method based on the thiol-derivatised probe, the whole coating procedure was performed outside the instrument and only at the end the chip was docked into the instrument. For this reason, the same probe was immobilised on the different flow cells (cells 1 and 2: probe P35S).

With this different immobilisation approach, the operating parameters, i.e. regeneration conditions, the hybridisation solution, etc., were optimised.

The regeneration conditions were changed and the working buffers (hybridisation buffer both as running and as dilution buffer for the target solutions) were spiked with 0.005% of Tween 20. In order to allow the reuse of the sensing surface, different concentrations of HCl and NaOH were tested as regenerating agents. Among them, HCl 2.5 mM (2 μl) showed the best regeneration capacity.

In order to test the specificity of the system, a non-complementary oligonucleotide 0.1 μM was used. The signal resulting from these non-specific oligonucleotides was negligible (ΔRU<1 RU).

The reproducibility of the measurements was evaluated over nine hybridisation cycles with the target P35S 0.1 μM, which resulted in a CV% of 1% for both cells (154±2 RU and 138±1 RU for cells 1 and 2, respectively).

In Fig. 4, the calibration curve obtained for target concentrations 0–0.5 μM is reported. Since the same P35S probe has been immobilised on both the flow-cells, in the figure both signals are taken into consideration. A linear range up to 25 nM with an experimental limit of detection for P35S target of 2.5 nM can be observed.

Regarding the stability of the chip, with the previously reported regeneration procedure, more than 100 cycles of hybridisation/regeneration could be performed on the same chip without affecting the sensitivity (data not reported).

Probe bovine. A calibration curve (0–0.5 μM) for bovine probe (Fig. 5) was obtained using the same experimental conditions as in the previous case. Similar results were observed in both cases: a linear range up to 25 nM and an experimental limit of detection for bovine target of 2.5 nM.

The reproducibility of the measurements was evaluated over 10 hybridisation cycles with the target bovine 0.1 μM. The resulting average frequency shifts were 146±6 and 152±7 RU for cells 1 and 2, respectively, with a CV% of 4% for both cells. The specificity of the system was tested employing a non-complementary sequence 0.1 μM; the signals resulting from these non-specific interactions were negligible (ΔRU<1 RU).

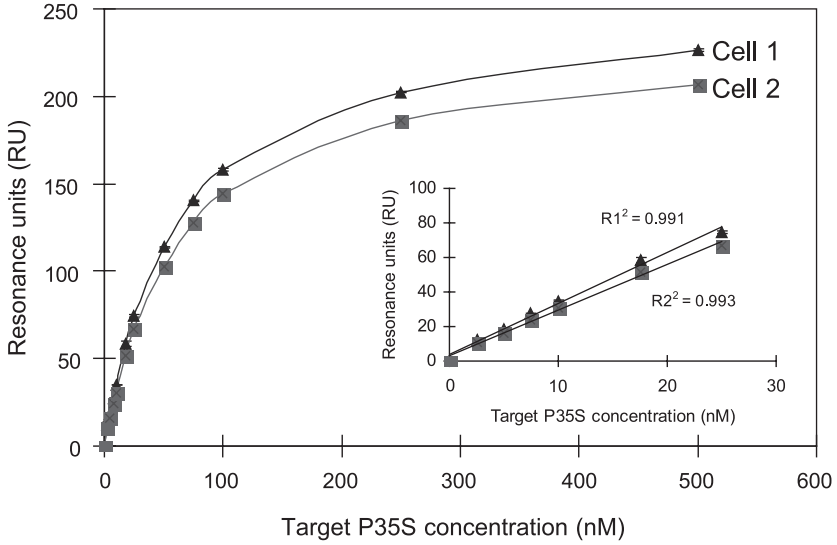

Fig. 4. Calibration curve for P35S obtained from thiol-derivatised probe/blocking thiol immobilisation procedure using BIACORE X™ instrument and zoom of the linear region (cell 1: probe P35S; cell 2: probe P35S).

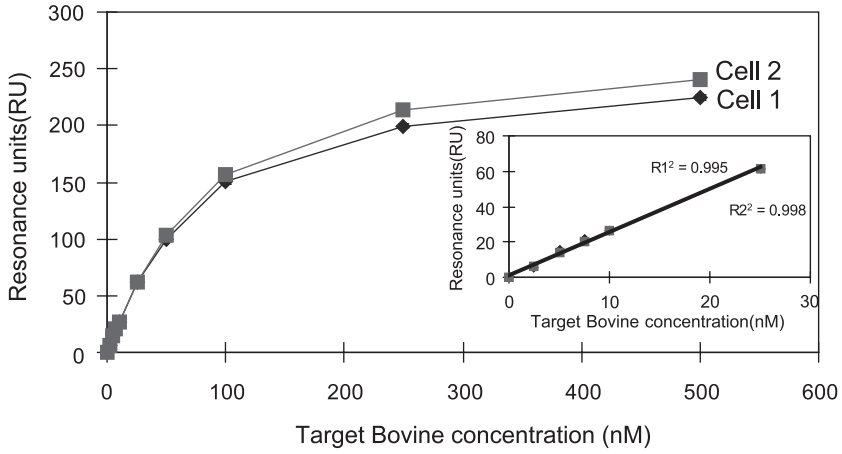

Fig. 5. Calibration curve for bovine obtained from thiol-derivatised probe/blocking thiol immobilisation procedure using BIACORE X™ instrument and zoom of the linear region (cell 1: probe bovine; cell 2: probe bovine).

Also in this case, more than 100 cycles of hybridisation/regeneration could be performed on the same chip without decreasing the sensitivity (data not shown).

3.1.2.3. Thiol-derivatised probe and blocking thiol procedure on Spreeta™

Probe P35S. Using the Spreeta™ device, the whole immobilisation procedure, except the cleaning step, has been conducted on the chip already inserted into the instrument. Due to the presence of only one flow-cell, only the specific P35S probe has been immobilised on the chip.

In order to evaluate the performance of the device, a calibration curve has been obtained with different concentrations of target P35S (0–1 μM) (Fig. 6). An experimental detection limit of 10 nM was obtained.

A non-complementary oligonucleotide 1 μM was tested to check the specificity of the system and non-detectable hybridisation signals were obtained from these experiments.

The reproducibility was evaluated performing six measurements on the same day with the target P35S 0.1 μM. An average hybridisation signal of (10.1±0.6)e−05 RI was obtained with a CV% of 6%.

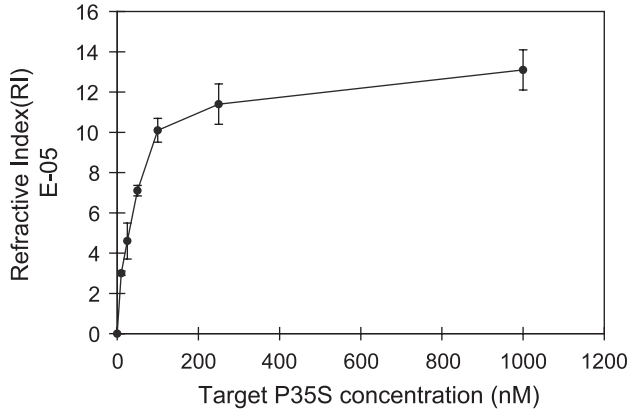

Fig. 6. Calibration curve for P35S obtained from thiol-derivatised probe/blocking thiol immobilisation procedure using SPREETA™ instrument.

The regeneration procedure chosen for this system was the one optimised using the Biacore X™ device with the same immobilisation method (HCl 2.5 mM). This treatment allowed up to 50 cycles of hybridisation (data not shown).

3.2. Hybridisation with PCR amplified DNA and digested genomic DNA

3.2.1. QCM device

Both the immobilisation procedures were applied to the analysis of PCR amplified DNA (P35S) and digested genomic DNA (bovine). The results are summarised in Fig. 7.

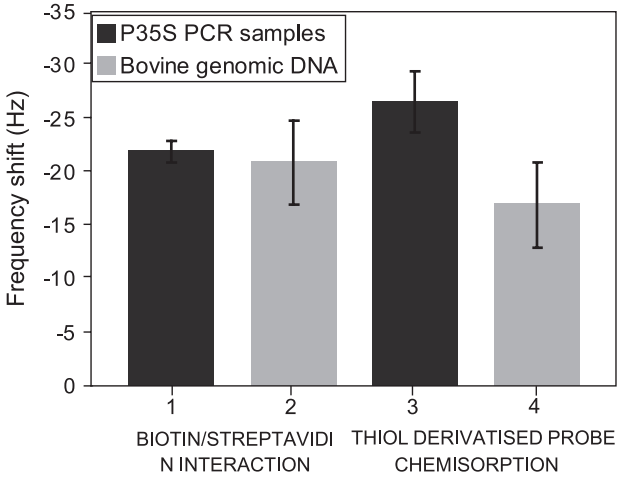

Fig. 7. Frequency shifts obtained from biotin-derivatised probe and thiol-derivatised probe/blocking thiol immobilisation procedures using QCM instrument and PCR amplified DNA and digested genomic DNA samples. In detail: (1) biotin-derivatised probe and PCR amplified DNA; (2) biotin-derivatised probe and digested genomic DNA; (3) thiol-derivatised probe and PCR amplified DNA; (4) thiol-derivatised probe and digested genomic DNA.

Table 1
Results obtained with the SPR devices (BIACORE X™ and SPREETA™) using thiolated probes, immobilised on the sensing surfaces

	Biacore X™			Spreeta™		
	ΔRU	S.D. ($n \geq 3$)	CV%	ΔRI	S.D. ($n \geq 3$)	CV%
P35S (PCR amplified DNA, 243 bp)	90	5	6	10.5e−05	0.7e−05	7
Bovine genomic DNA (enzymatically digested, 400–500 bp)	60	5	8	/	/	/

The PCR amplified DNA sample has been analysed with both SPR devices, immobilising on the surface the P35S probe. The enzymatically digested DNA sample has been tested only with the BIACORE X™ device, using the bovine probe. (1 RI=1e+06 RU).

The system was able to detect the complementary target sequence in both samples, differing in complexity. In particular, in amplified DNA, the target sequence is present alone in a high number of copies, while in the digested DNA the target sequence is present in a lower number of copies and it is also mixed with all the fragmented genomic DNA.

The calculated CV% are 4% and 11% for PCR samples (columns 1 and 3) and 19% and 23% for genomic digested DNA (columns 2 and 4). These differences can be explained taking into consideration the different complexity of the two samples.

The specificity of the system was tested with blank solutions (PCR mixture without the DNA template for P35S and the digestion solution without the DNA template for bovine DNA). Negative controls consisting in PCR amplified DNA that did not contain the P35S target sequence, (217-bp sequence of lectin gene) and digested genomic DNA from a different species (porcine DNA), were also tested. The obtained frequency shifts were negligible in all cases (<3 Hz).

3.2.2. SPR device

The results obtained with the SPR devices using thiolated probes are shown in Table 1.

P35S PCR samples and bovine genomic DNA have been tested with Biacore X™. In both cases, the system was able to detect the target sequence with a good reproducibility (CV%: 6% and 8%). As in the case of QCM, the specificity was tested with non-specific samples and blank solutions. The signals were negligible (<1 RU), confirming the specificity of the surfaces modified by direct chemisorption of thiolated probe. These results can be compared with those obtained with biotinylated probes reported in the previous papers published by this group [15,16,35].

P35S PCR samples have been tested with the Spreeta™ device, which showed similar performances (1 RI=1e+06 RU).

4. Conclusions

Different examples of DNA-based biosensors realised with two immobilisation procedures have been presented. The first method was based on the coupling of biotinylated oligonucleotide probes onto a streptavidin-coated dextran-modified surface and it was taken as reference method. The second was based on the direct coupling of thiolated probes (Probe-C_6-SH) to bare gold surfaces and it was proposed as alternative immobilisation method suitable for DNA-sensing.

Three different devices have been considered with the common characteristic of having a gold surface as sensing element: a quartz crystal microbalance (Seiko QCA922) and two surface plasmon resonance based devices (Biacore X™ and Spreeta™).

The application of the same coupling strategy to these different devices, using different sensor surface formats, has been demonstrated.

The systems were suitable for the analysis of small oligonucleotides, PCR fragments and more complex samples such as genomic digested DNA. The obtained results with both immobilisation strategies were comparable when analysing PCR or genomic DNA samples both with the SPR and the QCM devices.

These results demonstrate the applicability of the alternative immobilisation method for DNA sensing using devices with different sensor formats.

References

[1] B. Kasemo, Biological surface science, Surf. Sci. 500 (2002) 656–677.
[2] T.M. Herne, M.J. Tarlov, Characterization of DNA probes immobilized on gold surfaces, J. Am. Chem. Soc. 119 (1997) 8916–8920.
[3] S. Lofas, B. Johnsson, A novel hydrogel matrix on gold surfaces in surface plasmon resonance sensors for fast and efficient covalent immobilization of ligands, J. Chem. Soc., Chem. Commun. 21 (1990) 1526–1528.
[4] H. Miyachi, K. Yano, K. Ikebukuro, M. Kono, S. Hoshina, I. Karube, Application of chimeric RNA–DNA oligonucleotides to the detection of pathogenic microorganisms using surface plasmon resonance, Anal. Chim. Acta 407 (2000) 1–10.
[5] H. Morgan, D.M. Taylor, A surface plasmon resonance immunosensor based on the streptavidin–biotin complex, Biosens. Bioelectron. 7, 6 (1992) 405–510.
[6] M. Brigham-Burke, J.R. Edwards, D.J. O'Shannessy, Detection of receptor–ligand interactions using surface plasmon resonance: model studies employing the HIV-1 gp120/CD4 interaction, Anal. Biochem. 205 (1992) 125–131.
[7] B.C. Cunningham, J.A. Wells, Comparison of a structural and a functional epitope, J. Mol. Biol. 234 (1993) 554–563.

[8] R.J. Fisher, M. Fivash, J. Casas-Finet, J.W. Erickson, A. Kondoh, S.V. Bladen, C. Fisher, D.K. Watson, T. Papas, Real-time DNA binding measurements of the ETS1 recombinant oncoproteins reveal significant kinetic differences between the p42 and p51 isoforms, Protein Sci. 3 (1994) 257–266.
[9] S. Lofas, B. Johnsson, K. Tegendal, I. Ronnberg, Dextran-modified gold surface plasmon resonance sensors: immunoreactivity of immobilized antibodies and antibody–surface interaction studies, Colloids Surf., B Biointerfaces 1 (1993) 83–89.
[10] K. Kukanskis, J. Elkind, J. Melendez, T. Murphy, G. Miller, H. Garner, Detection of DNA hybridization using the TISPR-1 surface plasmon resonance biosensor, Anal. Biochem. 274 (1999) 7–17.
[11] C.E. Jordan, A.G. Frutos, A.J. Thiel, R.M. Corn, Surface plasmon resonance imaging measurements of DNA hybridization adsorption and streptavidin/DNA multilayer formation at chemically modified gold surfaces, Anal. Chem. 69 (1997) 4939–4947.
[12] N. Bianchi, C. Rutigliano, M. Tomassetti, G. Feriotto, F. Zorzato, R. Gambari, Biosensor technology and surface plasmon resonance for real-time detection of HIV-1 genomic sequences amplified by polymerase chain reaction, Clin. Diagn. Virol. 81 (1997) 199–208.
[13] G. Feriotto, M. Borgatti, C. Mischiati, N. Bianchi, R. Gambari, Biosensor technology and surface plasmon resonance for real-time detection of genetically modified roundup ready soybean gene sequences, J. Agric. Food Chem. 50 (2002) 955–962.
[14] C.M. Niemeyer, W. Burger, R.M.J. Hoedemakers, Hybridization characteristics of biomolecular adaptors, covalent DNA-streptavidin conjugates, Bioconjug. Chem. 9 (1998) 168–175.
[15] E. Giakoumaki, M. Minunni, S. Tombelli, I.E. Tothill, M. Mascini, P. Bogani, M. Buiatti, Combination of amplification and post-amplification strategies to improve optical DNA sensing, Biosen. Bioelectron. 19 (4) (2003) 337–344.
[16] E. Mariotti, M. Minunni, M. Mascini, Surface plasmon resonance biosensor for genetically modified organisms detection, Anal. Chim. Acta 453 (2002) 165–172.
[17] V. Silin, A. Plant, Biotechnological applications of surface plasmon resonance, Tibtech 15 (1) (1997) 353–359.
[18] M. Yang, H.C.M. Yau, H.L. Chan, Adsorption kinetics and ligand-binding properties of thiol-modified double-stranded DNA on a gold surface, Langmuir 14 (1998) 6121–6129.
[19] A.B. Steel, R.L. Levicky, T.M. Herne, M.J. Tarlov, Immobilization of nucleic acids at solid surfaces: effect of oligonucleotide length on layer assembly, Biophys. J. 79 (2000) 975–981.
[20] F. Song, F. Zhou, J. Wang, N. Tao, J. Lin, R. Vellanoweth, Y. Morquecho, J. Wheeler-Laidman, Detection of oligonucleotide hybridization at femtomolar level and sequence-specific gene analysis of the Arabidopsis thaliana leaf extract with an ultrasensitive surface plasmon resonance spectrometer, Nucl. Acids Res. 30 (14) (2002) 72.
[21] Y. Cho, S. Kim, G. Lim, S. Granick, A surface forces study of DNA hybridization, Langmuir 17 (2001) 7732–7734.
[22] M. Satjapipat, R. Sanedrin, F. Zhou, Selective desorption of alkanethiols in mixed self-assembled monolayers for subsequent oligonucleotide attachment and DNA hybridization, Langmuir 17 (2001) 7637–7644.
[23] T. Hianik, V. Gajdos, R. Krivanek, T. Oretskaya, V. Metelev, E. Volkov, P. Vadgama, Amperometric detection of DNA hybridization on a gold surface depends on the orientation of oligonucleotide chains, Bioelectrochemistry 53 (2001) 199–204.
[24] S. Tombelli, M. Mascini, C. Sacco, A.P.F. Turner, A DNA piezoelectric biosensor assay coupled with a polymerase chain reaction for bacterial toxicity determination in environmental samples, Anal. Chim. Acta 418 (2000) 1.
[25] S. Tombelli, M. Mascini, L. Braccini, M. Anichini, A.P.F. Turner, Coupling of a DNA piezoelectric biosensor and polymerase chain reaction to detect apolipoprotein E polymorphisms, Biosens. Bioelectron. 15 (7–8) (2000) 363–370.
[26] I. Mannelli, M. Minunni, S. Tombelli, M. Mascini, Bulk acoustic wave affinity biosensor for genetically modified organisms detection, IEEE Sens. J. 3 (4) (2003) 369–375.
[27] N.K. Chaky, K. Vijayamohanan, Review. Self-assembled monolayers as tunable platform for biosensor applications, Biosens. Bioelectron. 17 (2002) 1–12.
[28] A. Ulman (Ed.), An Introduction to Ultrathin Organic Films: From Langmuir-Blogget to Self Assembly, Academic Press, New York, 1991.
[29] L.H. Dubois, R.G. Nuzzo, Synthesis, structure, and properties of model organic surfaces, Annu. Rev. Phys. Chem. 43 (1992) 437–463.
[30] C.D. Bain, G.M. Whitesides, Modeling organic surfaces with self-assembled monolayers, Angew. Chem., Int. Ed. Engl. 28 (1989) 506–512.
[31] M. Minunni, S. Tombelli, S. Pratesi, M. Mascini, P. Piatti, P. Bogani, M. Buiatti, M. Mascini, A piezoelectric affinity biosensor for genetically modified organisms (GMOs) detection, Anal. Lett. 34 (2001) 6.
[32] K. Pietsch, U. Waiblinger, P. Brodmann, A. Wurzl, Screeningverfahren zur Identifizierung “gentechnisch veränderter” pflanzlicher Lebensmittel, Dtsch. Lebensm.-Rundsch. 2 (1997) 35–38.
[33] M. Pech, R.E. Streeck, H.G. Zachau, Patchwork structure of a bovine satellite DNA, Cell 18 (1979) 883–893.
[34] J. Sambrook, E.F. Fritsch, T. Maniatis (Eds.), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[35] R. Wang, S. Tombelli, M. Minunni, M.M. Spiriti, M. Mascini, Direct immobilisation of DNA probes for the development of SPR-based sensing. Biosens. Bioelectr. (in press).